(19) (CA) CANADIAN PATENT (12)

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ABSTRACT OF THE DISCLOSURE

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A test for lipase activity is provided wherein
trilinolein, or an equivalent as defined herein, is employed
in an emulsified form as a substrate. Reduced incubation
times and improved accuracy are obtained by providing an
excess of lipoxygenase to convert the linoleic acid product
of lipolysis to linoleic acid hydroperoxide which is measur-
able with great accuracy at low concentrations by spectro-
photometric or kinetic assay techniques, for example.

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The invention relates to the determination of lipase
activity in a biological or physiological fluid.

The usefulness of the determination of lipase
activity in physiological fluids, including serum and duodenal
fluid, for example, in early diagnosis of pancreatic disease
and monitoring the clinical course thereof has been generally
acknowledged in the past. However, technical difficulties as
well as problems with specificity and sensitivity of existing
lipase methods have generally hindered the more widespread
10   use of this enzyme as a primary laboratory indicator of
pancreatic function.

The most widely accepted methods for measuring
lipase activity in biological fluids usually employ olive oil
as a substrate and rely on titration, with standardized sodium
hydroxide of the fatty acids liberated during a 24 hour
incubation period. Some major disadvantages of these types
of methods include long incubation times, resulting in non-
linearity and reduced specificity; large sample requirements;
the requirement for highly concentrated and stable olive oil
20   emulsions, which are difficult and cumbersome to prepare; and
the difficulties of reproducing the end point in the tit-
ration of the extremely weak long-chain fatty acids. Colori-
metry of the free fatty acids produced by lipolysis of an olive
oil substrate (in the form of their copper soaps, after
extraction into a lipid solvent) permits shorter incubation
times (10-30 minutes), but involves many tedious manipulations
which limits its usefulness in a routine laboratory. The pro-
cedures described above, including variations thereof employ-
ing sensitive fluorescent pH indicators, for example, in
30   addition to the described difficulties, lack the desired
sensitivity and precision in the normal range of lipase
activities.

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In addition to the above described methods, turbidi-
metric methods of lipase analysis have been employed in the
past, but the substrate concentrations used are suboptimal to
permit the rate of substrate clearance to be measured in a
reasonably accurate photometric range and initial increases
in absorbance and nonlinear absorbance changes throughout
the entire reaction period have been reported for patients'
samples, indicating problems with some of these procedures.
In addition to the olive oil substrate, several synthetic
10   soluble chromogenic and fluorogenic substrates, mostly mono-
esters of long-chain fatty acids, have been proposed for use
in the measurement of lipase activity in serum. However,
it has been conclusively shown that pancreatic lipase does not
act on soluble esters but is active only when adsorbed at an
oil/water interface. Thus, the nonemulsified state of these
types of substrates indicates limited usefulness as analytical
tools for measuring pancreatic lipase activity. Lipase
activity has also been determined by radial enzyme diffusion,
based on measurement of the cross-sectional area of the
20   clearing of an olive oil emulsion suspended in buffered
agarose gel. The disadvantages of this method are that two
hour incubation times are required, accurately measuring
small diameters of the clear zone is difficult, and it is
necessary to calibrate with secondary standards.

Thus, a method for the determination of lipase
activity in biological fluids which has reduced incubation
time, can employ relatively small amounts of sample fluids,
and which is highly specific and sensitive is desirable.
Further, a lipase activity measurement procedure which can
30   be performed simply, using readily available laboratory
equipment, would be advantageous.

The method of the present invention overcomes the disadvantages of the lipase activity tests briefly described above by employing a coupled enzymatic reaction sequence to convert the primary product of lipolysis into a chemical compound that may be measured with greater speed and ease and with greater sensitivity, precision, and accuracy than is possible in the analysis of free long-chain fatty acids by either titrimetric or indirect photometric methods following solvent extraction.

10   According to the invention there is provided a method for determining the lipase activity of a biological fluid comprising: contacting a sample of a biological fluid with an emulsified substrate effective in the presence of lipase to produce fatty acid by lipolysis, contacting any fatty acid produced with lipoxygenase in the presence of oxygen to convert any fatty acid produced by said lipolysis to its acid hydroperoxide, said lipoxygenase being added in an amount to effect the conversion at a rate sufficient to ensure that lipolysis is the limiting reaction, and measuring

20   the production of the acid hydroperoxide as a measure of the lipase activity of the biological fluid.

According to another aspect of the invention there is provided a reagent for determining the lipase activity of a biological fluid comprising: an emulsified substrate effective in the presence of lipase to produce fatty acid by lipolysis, and lipoxygenase in an amount effective, in the presence of oxygen, to convert the acid produced by lipolysis to its acid hydroperoxide at a rate sufficient to ensure that lipolysis is the limiting reaction.

30   In a particular embodiment there is provided a test system for determining the lipase activity of a biological fluid comprising the reagent of the invention and means for

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measuring the production of linoleic acid hydroperoxide
as a measure of the lipase activity of the biological fluid.

Basically, in a particular embodiment the method
comprises adding a sample of biological fluid to a substrate
emulsion to thereby induce lipolysis. The substrate employed
is preferably trilinolein but can be an equivalent thereof
as further defined hereinbelow. Either at the same time or
shortly thereafter, an amount of lipoxygenase is added which
is effective to convert the fatty acid produced by lipolysis
to its acid hydroperoxide, the conversion being effected
at a rate substantially greater than that at which the
acid is produced. The hydroperoxide can then be measured
by, for example, colorimetry, uv-spectrophotometry, or kinetic
assay techniques.

A lipoxygenic endpoint assay requires only about 15
minutes and the kinetic version of the tests, which usually
employs an oxygen electrode, only a few minutes for completion
which makes the assay well suited for an emergency procedure.
Further, increased sensitivity is achieved by the coupled
enzymatic reaction as compared to the standard lipase
methods that measure the weak long-chain fatty acids produced
during incubation of the sample with olive oil or triolein
emulsions by titration with alkali, thus permitting the use of
microsamples which makes the procedure ideally applicable to
pediatric applications.

The rapid enzymatic micromethod for specific deter-
mination of the diagnostically important enzyme lipase (glycerol
ester hydrolase, E.C. No. 3.1.1.3) of the present invention
employs a coupled enzymatic reaction sequence whereby the free
fatty acid, particularly linoleic acid produced by lioplysis
of an emulsified substrate, for example, a trilinolein
substrate or a substrate formulation employing one of the other

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triglycerides set forth in detail below, is converted to its
more easily identifiable acid hydroperoxide. The conversion is
accomplished by providing an excess amount of lipoxygenase (also
known as lipoxidase, linoleate:oxygen oxidoreductase E.C.1.99.2.1)
so that the reate of formation of the acid by lipolysis is the
rate limiting step of the coupled enzymatic reaction.

The basic coupled enzymatic reaction sequence can be
schematically depicted in the following manner, by reference to
the production of linoleic acid as the fatty acid:

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    (1) Trilinolein <u>Lipase</u> $\longrightarrow$ 1, 2 Dilinolein + Linoleic Acid

    (2) Linoleic Acid + O_2 <u>Lipoxygenase</u> $\longrightarrow$ Linoleic Acid
                                                Hydroperoxide

Because an excess amount of lipoxygenase is employed, the
rate of formation of linoleic acid, which is directly pro-
portional to the activity of lipase under the employed
conditions of substrate saturation, may be measured by the
rate of production of linoleic acid hydroperoxide, or the
rate of consumption of oxygen. Suitable reaction conditions
for the coupled enzymatic reaction sequence are 30°C at a

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pH of 8.8. Under these conditions, the incubation time for
the coupled enzyme reaction is about 10 minutes and the
reaction can be stopped by the addition of alcohol. Thus,
when lipoxygenic endpoint assay techniques are employed, the
entire procedure takes only about 15 minutes, and when kinetic
assay techniques are employed the actual measurement of lipase
activity takes place during the incubation period. In com-
parison, the standard titrimetric olive oil procedures
typically require from 3 to 24 hours of incubation to generate
adequate amounts of titratable acid and the continuous sampling

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version of these types of methods requires a pH-stat instru-
ment which is generally unavailable to most clinical
laboratories. The coupled enzyme reaction sequence set forth

above is also superior to the presently available methods for measuring lipase activity in that the soluble substrates used, for example, trilinolein and its equivalents described hereinbelow are not hydrolized by the various carboxylesterases and therefore do not yield false positive results in cases where these non-specific esterases are elevated in body fluids.
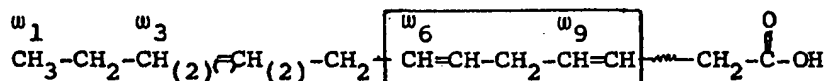
The specific enzymatic measurement of linoleic acid rather than of total acidity also eliminates the interference by proteolytic enzymes or any other non-specific H^+ producing or consuming side-reactions. The coupled enzymatic reaction also provides for a drastic increase in sensitivity when compared to standard lipase methods that measure the weak long-chain fatty acids produced during incubation of the sample with olive oil emulsions by titration with alkali. This increase in sensitivity permits the use of microsamples, which makes the test well suited for pediatric patients.

A further advantage of the lipase method of the present invention is that preparation of the substrate emulsion can be performed rapidly and in a technically simple and non-critical manner since adequate oil/water interface is easily obtained when microsamples are employed. This should be contrasted with the preparation of olive oil emulsions for the existing standard titrimetric methods which preparation is a technically involved cumbersome procedure requiring the addition of an emulsifier such as gum acacia, in order to obtain a stable emulsion of high oil/water interfacial area, which is crucial for the success of the method.

While the naturally occuring glycerol ester trilinolein is the preferred substrate material, it will be apparent to one skilled in the art that other natural or synthetic, simple or mixed triglycerides or triacylglycerols
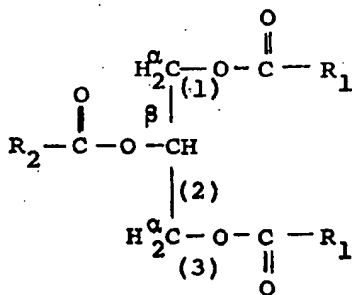
can be employed. Basically, the requirements of other useful natural or synthetic, simple or mixed triglycerides are that the component fatty acids, esterified to glycerol in 1,3-(or α) position meet the structural requirements for a good substrate for the enzyme lipoxygenase, i.e. result in high rates of formation of hydroperoxide in the presence of oxygen. Present knowledge of the enzyme suggests that a methylene interrupted cis-diene system with double bonds extending from carbon atom No. 6 to 7 (ω6) and from carbon atom No. 9 to 10 (ω9) count-

10   ing from the methyl end of the fatty acid must be present according to:

$$CH_3\text{-}CH_2\text{-}\overset{\omega_1}{CH}_{(2)}\text{-}\overset{\omega_3}{CH}_{(2)}\text{-}CH_2\text{-}\boxed{\overset{\omega_6}{CH}=CH\text{-}CH_2\text{-}\overset{\omega_9}{CH}=CH}\text{-}\!\sim\!\text{-}CH_2\text{-}\overset{O}{\overset{\|}{C}}\text{-}OH$$
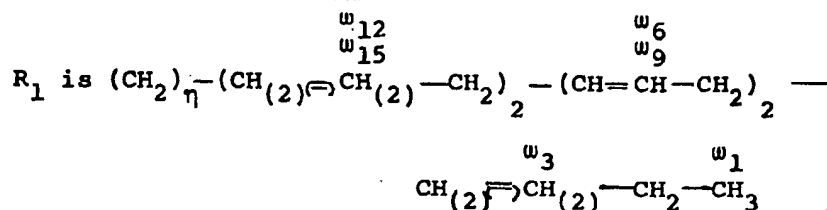
Other double bonds (e.g. ω3, ω12, ω15 .....) may or may not be present.

The general structure for suitable triglycerides may be depicted schematically as follows:

$$\begin{array}{c}
\quad\quad\quad\quad\quad\quad\quad\quad O \\
\quad\quad\quad\quad\quad\quad\quad\quad \| \\
\quad\quad\quad\quad H_2\overset{\alpha}{C}\!\!-\!\!O\!-\!C\!-\!R_1 \\
\quad\quad\quad O \quad |(1) \\
\quad\quad\quad \| \quad \overset{\beta}{\underset{}{|}} \\
R_2\!-\!C\!-\!O\!-\!CH \\
\quad\quad\quad\quad\quad |(2) \\
\quad\quad\quad H_2\overset{\alpha}{C}\!-\!O\!-\!C\!-\!R_1 \\
\quad\quad\quad\quad (3) \quad \| \\
\quad\quad\quad\quad\quad\quad O
\end{array}$$

$$R_1 \text{ is } (CH_2)_\eta - \overset{\omega 12}{\underset{\omega 15}{(CH_{(2)}}}CH_{(2)} - CH_2)_2 - \overset{\omega 6}{\underset{\omega 9}{(CH}} = CH - CH_2)_2 -$$

$$\overset{\omega 3}{CH_{(2)}}CH_{(2)} - \overset{\omega 1}{CH_2} - CH_3$$

η is 1, 3 or 5 for naturally occurring and 1, 2, 3, 4 or 5

for synthetic fatty acids.

R_2 is R_1 for the preferred simple triglyceride substrates.

Otherwise stated R_1 may be a group of formula

$$(CH_2)_\eta - A_2(CH=CH-CH_2)_2 - A - CH_3$$
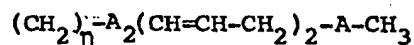
in which A is $-CH=CH-CH_2-$ or $-CH_2-CH_2-CH_2-$; and η is an

integer from 1 to 5.

10   R_2 may be any saturated or (poly)unsaturated fatty

acid alkyl chain in case of mixed triglyceride substrates.

This is so because the 2 (or β) esterlinkage is not

attacked by lipase during the initial reaction phase, and con-

sequently, there are no specific structural requirements for

the fatty acid bound in this position.

          Trilinolein, or an equivalent triglyceride of the

above specified structural formula, can be prepared in

emulsified form relatively easily for use as a substrate with

which lipase will conjugate according to the coupled enzymatic

20   reaction sequence of the invention. For example, trilinolein,

which is available in 100 mg/ampules, can be dissolved in an

ethanol/acetone solvent and stored at 0-4°C to form a substrate

stock solution which can be emulsified by blending with a

working buffer which provides the proper pH for the enzymatic

reactions. A suitable working buffer can be prepared from

sodium deoxycholate dissolved in tris(hydroxymethyl)amino-

methane (hereinafter sometimes referred to as Tris) with

sufficient hydrochloric acid to adjust the pH to the desired

level. Alternatively, barbital buffer may be substituted for
Tris. Substrate emulsions can be prepared by mixing the work-
ing buffer with the substrate stock solution and blending, at
maximum speed, in a commercial blender for about 5 minutes.
Preferably the substrate emulsions are prepared freshly each
day.

Lipoxygenase (linoleate:oxygen oxidoreductase,
E.C. 1.99.2.1) necessary to perform the specific conversion
of the linoleic acid produced by lipolysis to linoleic acid

10   hydroperoxide, can be prepared, in stock solution form, by
admixing lyophilized lipoxygenase of sufficient activity with
the appropriate volume of working buffer (described above with
relation to the substrate emulsion) to obtain the stock
solution having an activity of 1.40×10^6 S.U.*/ml ($\simeq 168,000$
I.U./ml at $25°$C). This lipoxygenase stock solution should be
stored at temperatures at from about $0°$ to about $4°$. A
lipoxygenase working solution having an activity of about
140,000 S.U./ml ($\simeq 16,800$ I.U./ml at $25°$C) can then be pre-
pared by diluting the lipoxygenase stock solution 1:10 with

20   more working buffer and should be prepared freshly each day.
The coupled enzyme reaction sequence of the present invention
is made possible by the high specificity of lipoxygenase for
free linoleic acid and the similarity of the pH optima of both
enzymes (pH 8.8 for lipase, pH 8.3 for lipoxygenase). The
lipoxygenase employed in the present invention can be isolated

* Definition of spectrophotometric unit (S.U.): One (1) S.U. will
cause an increase in absorbance at 234nm (ΔA_{234}) of 0.001 at $25°$C
when linoleic acid is the substrate in 3 ml volume at pH 9.0
(1 cm light path). 1 S.U. is equivalent to the oxidation of

30   0.12 µ mole of linoleic acid, i.e. 0.12 International Units
(I.U.B. units or I.U.) at $25°$C.

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from soybean-flour extracts.

While any biological fluid suspected of containing the enzyme lipase may be employed with the substrate emulsion, and lipoxygenase working solution set forth above to perform the coupled enzymatic reaction sequence of the present invention, the biological fluids of greatest interest are blood serum and duodenal fluid. Because of the extreme sensitivity of the test procedure of the subject invention, microsamples of blood serum and duodenal aspirate can be

10   employed. For example, 10 µl of serum sample (20 µl with test blank) can be employed in most cases. A like amount of diluted duodenal aspirate can be employed.

Thus, the above described reagents, that is substrate emulsion, lipoxygenase working solution and biological sample fluid are all the reagents necessary to form the coupled enzymatic reaction sequence of the present invention. Of course, other reagents will be useful depending upon the analytical method employed to determine lipase activity. However, for example, when kinetic assay techniques are

20   employed which measure directly the rate of oxygen consumption by the reaction between linoleic acid and oxygen (see equation 2 above) no other reagents need be employed.

The kinetic variables which influence the coupled enzymatic reaction of the subject invention, including substrate concentration, effects of sample matrix, effect of blending times and speeds on the substrates, effect of bile salts, effects of pH, effect of temperature, effect of lipoxygenase activity and effect of concentration of oxygen in the system were studied in order to better understand the

30   operating parameters of the system. The results of these studies will aid one skilled in the art in the practice of the subject invention.

With regard to substrate concentration, it should be noted that since the lipase acts on the oil/water interface only, the interfacial area of the substrate/buffer emulsion takes the place of the substrate concentration in the kinetic equation for homogeneous systems. However, if the substrate is insoluble in water, and the degree of emulsification is identical for all substrate concentrations used, the interfacial area increases linearly with increasing substrate concentration. Because of the technical difficulties of measur-

10   ing accurately the interfacial area of emulsions, the conditions of emulsification were kept constant throughout the study and the reaction rates were expressed as a function of the trilinolein concentrations. Basically, with respect to trilinolein, it was determined that at substrate concentrations of less than about 1.0×10^{-4} mol/liter there is insufficient substrate for saturation of the enzyme and at substrate concentrations above approximately 4×10^{-4} mol/liter there is an apparent decrease in the maximum velocity of the substrate-lipase reaction, indicating some form of substrate inhibition.

20   The effect of the sample matrix was studied by comparing serial dilutions of pancreatitis serum with heat-deactivated human serum and solutions of 10 grams of bovine serum albumin in 1 liter of working buffer. The differences in the sample matrix were not significant for lipase activities up to 1,000 U/liter. Apparently, at the high substrate concentration chosen for the assay, the velocity of the reaction is at a maximum whether the samples have been diluted or not and is equal for identical lipase activities, independent of the sample matrix.

30   The effect of blending times and speeds in preparation of the substrate emulsion was also investigated. Basically, it was found that substrates prepared simply by

vortex-mixing trilinolein for 3 to 5 minutes with the sub-
strate buffer led to data indicating inadequate interface
area or instability of the emulsion, or both. It was deter-
mined that the best results were obtained with homogenized
substrates, prepared by blending the chilled working buffer
in a commercial blender at a maximum speed with trilinolein
for 3 to 5 minutes. Blending for more than 5 minutes does
not apparently further increase either the linear range or the
stability of the substrate and may result in undesirable heat-
ing of the substrate. Substrates prepared by the methods
suggested above are stable for three to four days, if kept
refrigerated.

The effect of various concentrations of bile salts,
and specifically deoxycholate on the overall reaction rate
was studied. For both normal and pancreatitis serum samples
maximum activity was observed at 3.6 mmol of deoxycholate per
liter. Other bile salts (glycocholate, taurocholate) have a
similar effect.

The effect of pH on the rate of trilinolein hydrolysis
of both normal and pancreatitis serum was studied in the range
of 7.6 to 9.4 at 30°C. A trilinolein substrate, prepared in
50 mmol/liter at 25°C, was divided into several aliquots,
which were adjusted individually by dropwise addition of
12 mol/liter HCl to the desired pH values. The results of
these tests indicate that lipase of normal serum loses its
activity faster than that in diluted pancreatitis serum on
either side of the pH optimum which is about 8.8 at 30°C for
both samples.

The effect of temperature on the rate of trilinolein
hydrolysis was studied in the range between 20 and 40°C. The
pH was maintained at exactly 8.8 at all reaction temperatures,
by computing the corresponding pH at room temperature from the

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temperature coefficient of Tris buffer (0.025/°C), and adjusting the substrate pH values at 25°C accordingly by dropwise addition of 12 mol/liter HCl, or 10 mol/liter NaOH. The study shows that between 37 and 40°C an actual decrease in activities is observed, indicating progressive heat denaturation of either lipase or lipoxygenase, or both. These studies indicate that 30°C should be the recommended reaction temperature for the assay of the present invention.

In order to determine the degree of saturation of lipoxygenase, diluted pancreatitis serum was analyzed under the conditions of the assay of the present invention, using different lipoxygenase activities. At lipoxygenase activities of 1000 S.U./liter of substrate ($\simeq$ 120 I.U./liter at 25°C), the lipase-catalyzed hydrolysis of trilinolein apparently becomes rate limiting. The actual lipoxygenase activity used in the final assay is about 1400 S.U./liter ($\simeq$ 168 I.U./liter) in order to compensate for partial loss of enzyme activity by potential serum inhibitors of this enzyme. Thus, lipoxygenase activities of at least 1,000 S.U./liter of substrate (120 I.U./liter at 25°C) are necessary in order that the conversion of trilinolein to linoleic acid by lipase be the rate limiting reaction.

Finally, the effect of the concentration (partial pressure) of oxygen was studied. According to the second equation of the coupled enzymatic reaction sequence of the present invention, one mole of oxygen is consumed for each mole of linoleic acid converted to its hydroperoxide. In order to determine whether or not atmospheric conditions would provide sufficient oxygen to insure the reaction rate would remain unaffected, reactions using atmospheric conditions were compared to those carried out under a continuous

stream of pure oxygen, using a preoxygenated buffer. No

difference in either the velocity or the linearity of the

reaction with respect to time and activity were noted for

the latter type reactions, thus indicating that the test

can be carried out with non-oxygenated buffer in open test

tubes in normal laboratory atmosphere.

<u>EXAMPLE PROCEDURES</u>

In order to more fully illustrate the present

invention, example procedures are set forth below wherein

10 colorimetric, spectrophotometric, and kinetic assay techniques

are employed in combination with the coupled enzymatic reaction

sequence set forth above to determine lipase activity. It is

not intended that the scope of the present invention be

limited to these methods of analysis, and they are set forth

as exemplary only.

The starting materials, and reagents prepared there-

from, are set forth below and are examplary of those which

can be employed. Modifications thereof will be apparent to

one skilled in the art:

20 Sodium deoxycholate, AR.

Acid-alcohol solution, 2.62 g/liter. Dilute 6.0 ml

of 12 mol/liter hydrochloric acid (AR) to 1 liter with

absolute ethanol (AR).

Ferrous ammonium sulfate (AR), 0.13 mol/liter.

Dissolve 250 mg in 5.0 ml of hydrochloric acid, 30 g/liter.

Ammonium thiocyanate (AR), 2.63 mol/liter.

Dissolve 20 g in deionized water, dilute to 1 dl.

Lipoxygenase (linoleate:oxygen oxidoreductase,

E.C. 1.99.2.1.), activity, 165,000 S.U./mg ($\approx$ 19,800 I.U./mg

30 at 25°C.)

Lipoxygenase stock solution, 1.40×10^6 S.U./ml.
Dissolve 85 mg in 10.0 ml of working buffer. Store 0.1 ml
aliquots at 0-4°C.

Lipoxygenase working solution, 140,000 S.U./ml.
($\simeq$ 16,800 I.U./ml at 25°C). Dilute a 0.1 ml aliquot of
stock solution with 0.9 ml of working buffer. Prepare freshly
each day.

Trilinolein, 100 mg/ampule.

Trilinolein stock solution, 20 mg/ml. Dissolve the
10 contents of a 100 mg ampule in 5.0 ml of ethanol/acetone
(3/2 by vol.) Store at 0-4°C.

Working buffer. Dissolve 1.5 g (3.6 mmol) of
sodium deoxycholate in 1 liter of tris(hydroxymethyl)amino-
methane (Tris), 50 mmol/liter. Adjust the pH to 8.9 at 25°C
by dropwise addition of hydrochloric acid, 12 mol/liter.
Store at 2-6°C.

Substrate emulsion, 0.34 mmol/liter. Transfer
50 ml of the working buffer to the blender vessel, add 0.75
ml of trilinolein stock solution, blend at maximum speed
20 for 5 min. Prepare freshly each day.

Linoleic acid, 1 g/ampule.

Linoleic acid stock solution, 36 mmol/liter.
Dissolve the contents of a 1 g ampule in 1 dl of absolute
ethanol. Store at 0-4°C.

Linoleic acid working standard, 50 µmol/liter.
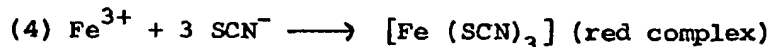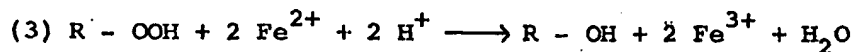Dilute 0.14 ml of stock solution to 1 dl with working buffer.

Solutions for linoleic acid standard curve. Dilute
280 µl of stock solution to 1 dl with working buffer, 100
µmol/liter. Serially dilute this standard with working
30 buffer to yield standards of 75, 50 and 25 µmol/liter.

Control sera.

Bovine Serum Albumin, 1 g/dl in deionized water.

Colorimetric Endpoint Assay

The amount of linoleic acid hydroperoxide produced by the coupled enzymatic reaction sequence of the invention within a 10 minute incubation period can be measured photometrically at 480 nm after dissolving the reaction mixture in acid alcohol, using the oxidation of ferrous to ferric ion by hydroperoxide and the detection of the ferric ion by thiocyanate according to the following equations:

(3) $R - OOH + 2 Fe^{2+} + 2 H^+ \longrightarrow R - OH + 2 Fe^{3+} + H_2O$

(4) $Fe^{3+} + 3 SCN^- \longrightarrow [Fe (SCN)_3]$ (red complex)

Lipase activity is computed in IUB units, with use of primary linoleic acid standards that are submitted to the same reaction sequence. The contribution of traces of free linoleic acid and of lipid peroxides, present in commercial batches of trilinolein, is subtracted in a substrate blank, the contribution of endogenous and exogenous sample constituents is compensated for in individual test blanks, and the contribution of the color reagents is subtracted using reagent blanks.

The following procedure can be employed to perform the colorimetric endpoint assay embodiment of the present invention.

(1) Label a series of test tubes, two for each patient's serum (T and TB) plus one each for a standard (S), "substrate blank" (SB), and "reagent blank" (RB).

(2) To the tubes labeled T and SB, add 1 ml of substrate emulsion. To the S tube add 1 ml of the 50 μmol/ liter linoleic acid standard. To the tubes labeled TB and RB, add 1 ml of working buffer.

(3) Place all the tubes in a 30°C waterbath for 5 minutes.

(4) In an exactly timed sequence (a 15 second interval is recommended), add 10 μl of serum, add 10 μl of lipoxygenase working solution, in that order, to the tubes labeled T and TB, vortex-mixing for 5 seconds after each addition, and replace it in the waterbath. To the tubes labeled S and SB add 10 μl of lipoxygenase only, and vortex-mix for 5 seconds replacing them in the waterbath.

(5) After exactly 10 minutes, and in the same timed interval, remove each tube from the waterbath and add 6 ml of acid/alcohol reagent from a dispenser and vortex-mix briefly. It should be noted that because the absorbance of the test blanks does not change measurably during the 10 minute incubation period, it is not necessary to adhere to a timed sequence for the test blanks, and the acid-alcohol reagent may be added to the TB tubes at any time while the T tubes are still incubating.

(6) Add 20 μl of ferrous ammonium sulfate reagent to all tubes and vortex-mix briefly.

(7) Add 100 μl of ammonium thiocyanate reagent to all tubes and vortex-mix briefly.

(8) Measure the absorbance of each sample at 480 nm against the reagent blank (RB).

The enzymatic activity of the serum sample can be calculated using the following formula:

$$\text{Lipase activity (U/liter)} = \frac{\Delta A_T}{A_s} \times 500 \text{ where}$$

$$\Delta A_T = A_T - A_{TB} - A_{SB}$$

The above formula was derived from the following known factors:

sample dilution factor: 100 (10 μl ⟶ 1 ml)

incubation time: 10 minutes

linoleic acid standard: 50 μmol/liter

50 μmol/liter substrate is equivalent to 5 mmol/liter

sample

5 mmol/liter per 10 min is equivalent to 500 μmol/

liter per min (500 U/liter)

Alternatively, the concentration of linoleic acid

corresponding to ΔA_T can be read from the linoleic acid

standard curve in μmol/liter and multiplied by 10 to obtain

the lipase activity in U/liter.

10   UV - Spectrophotometric Endpoint Assay

The linoleic acid hydroperoxide concentration can

be measured directly in an ethanol diluted reaction mixture

via its UV absorption at 234 nm (E_{234} ≃ 23,000 at 37°C) using

a narrow-band UV - Spectrophotometer. This procedure is

recommended especially for the assay of lipase activity in

duodenal aspirates.

Duodenal aspirates can be prepared for use as

samples in the method of the present invention by centrifuging

specimens obtained by incubation at 0-8°C at 4,000 g for

20   approximately 10 minutes to remove suspended particles. The

duodenal aspirate is then diluted 40-80 fold with a 10 g/liter

solution of bovine serum albumin.

Next, the procedure set forth in steps (1) through

(5) above with respect to the colorimetric assay procedure,

are repeated substituting the diluted duodenal juice for

the serum sample.

After the above steps and procedures have been per-

formed, the absorbance of each sample is read in 1 cm quartz

cuvets at 234 nm against the reagent blank. Lipase activity

30   in the unknown sample can then be calculated using the same

equation set forth above with respect to the colorimetric assay

technique.

Alternatively, the concentration of linoleic acid hydroperoxide (C_{LAH}) corresponding to ΔA_T may be determined directly (without use of standards) once the molar extinction coefficient of linoleic acid hydroperoxide at 234 nm (E_{234}) has been measured with the spectrophotometer employed. This calculation can be performed using the following equations:

$$C_{LAH}\left[\frac{\mu mol}{liter}\right] = \frac{\Delta A_T \cdot X \: 10^6}{E_{234}*}$$

Lipase activity (U/liter) $= C_{LAH} \: X \: 10**$

* E_{234} (37°C) $\doteq$ 23,000

** $\dfrac{\text{Dilution factor of sample}}{\text{Incubation time (min)}} = \dfrac{100}{10} = 10$

Kinetic Assay Using a pO_2 Electrode

Using this method of analysis the reaction is monitored by the decrease in oxygentension (pO_2) as linoleic acid produced during lipolysis reacts with an equimolar amount of dissolved oxygen to form linoleic acid hydroperoxide, using a pO_2 electrode. Lipase activity is computed with the initial rate of decrease of pO_2 in the reaction mixture after addition of sample lipoxygenase to the trilinolein emulsion substrate. The apparatus necessary to perform this type of analysis includes micro pO_2 electrodes suitable for blood gas analysis with associated signal amplifier and read-out device (strip-chart recorder, printer, etc.). A thermostat controlled reactionchamber (30°C), containing the sensing element of the oxygenelectrode and a magnetic stirrer. The assay is carried out in the following manner:

(1) 1 ml of the previously aerated substrate emulsion is pipetted into the reaction chamber. The electrode is allowed to stabilize. The recorder is set to maximum reading.

(2) 10 μl of sample (serum, duodenal aspirate,
control, etc.) and 10 μl of lipoxygenase working solution are
added with a micro-pipet. The magnetic stirrer is activated
for 5-10 seconds.

(3) The decrease in oxygen tension is recorded as
the reaction proceeds for approximately 3-6 minutes.

(4) The slope of the pO_2/time curve is measured
as soon as it becomes linear.

(5) The system is zero-rated by repeating steps

10   1-4 as described, but omitting the sample addition of step 2.

(6) The rate obtained using the substrate blank
(SB) is subtracted from the rate obtained with the test
sample (T) to obtain the net oxygen consumption rate
($\Delta r_T = r_T - r_{SB}$).

(7) A suitable lipase reference standard is then
assayed the same way as the unknown sample and its net oxygen
consumption rate is calculated (Δr_{ref}). The lipase activity
in the unknown test sample can be computed as follows:

20   Lipase activity (U/liter) = $\dfrac{\Delta r_T}{r_{ref}}$ X Lipase activity of
reference standard
(U/liter)

Alternatively, the lipase activity in the unknown
sample may be calculated directly from the decrease in oxygen
tension per minute after calibration of the system with
primary linoleic acid standards (1 μmol of linoleic acid will
consume 1 μmol oxygen under the conditions of this assay).
1 (IUB) Unit results in the consumption of 1 μmol of O_2 per
minute under the conditions of the assay.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1.   A method for determining the lipase activity of a biological fluid comprising

contacting a sample of a biological fluid with an emulsified substrate effective in the presence of lipase to produce fatty acid by lipolysis,

contacting any fatty acid produced with lipoxygenase in the presence of oxygen to convert any fatty acid produced by said lipolysis to its acid hydroperoxide, said lipoxygenase being added in an amount to effect the conversion at a rate sufficient to ensure that lipolysis is the limiting reaction,

and measuring the production of the acid hydroperoxide as a measure of the lipase activity of the biological fluid.
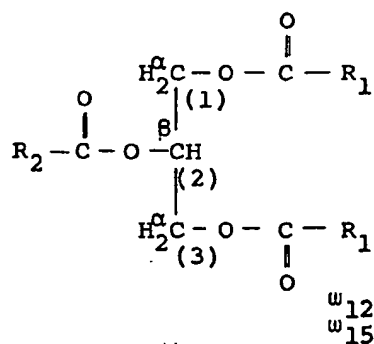
2.   A method according to claim 1, wherein said substrate is a triglyceride substrate.

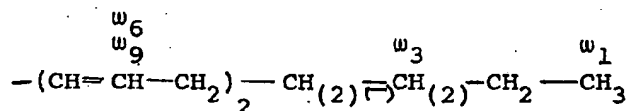3.   A method for the specific determination of lipase activity in a biological fluid comprising:

a)   adding a sample of said biological fluid to an emulsified substrate, said substrate having the structural formula:

$$H_2^{\alpha}C - O - \overset{\overset{O}{\|}}{C} - R_1 \quad (1)$$

$$R_2 - \overset{\overset{O}{\|}}{C} - O - \overset{\beta}{C}H \quad (2)$$

$$H_2^{\alpha}C - O - \overset{}{C} - R_1 \quad (3) \quad \underset{\|}{\overset{}{O}}$$

wherein R_1 is $(CH_2)_\eta \overset{\omega 12}{\overset{\omega 15}{-}}(CH_{(2)}\rightleftharpoons)CH_{(2)} - CH_2)_2 -$

$$-(CH \rightleftharpoons CH - CH_2)_2 \overset{\omega 6}{\overset{\omega 9}{-}} CH_{(2)}\rightleftharpoons\overset{\omega 3}{CH}_{(2)} - CH_2 \overset{\omega 1}{-} CH_3$$

η is an integer of 1 to 5, and R_2 is a member selected from the group consisting of R_1 and saturated or (poly)unsaturated fatty acid alkyl chains;

b)   simultaneously, or immediately thereafter, adding lipoxygenase, in the presence of oxygen, said lipoxygenase being added in an amount effective to convert the fatty acid produced by lipolysis to its acid hydroperoxide at a rate sufficient to ensure that lipolysis is the limiting reaction; and

c)   measuring the production of said acid hydroperoxide.

4.   A method according to claim 3, wherein the substrate is trilinolein.

5.   A method according to claim 3, carried out at a pH of about 8.8 and at a temperature of about 30°C.

6.   A method according to claim 3, 4 or 5, wherein the activity of said lipoxygenase is at least about 1,000 S.U./ liter of substrate.
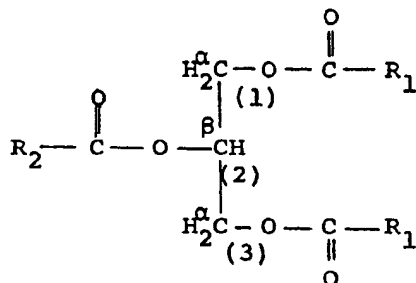
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7.   A method according to claim 3, 4 or 5, wherein the activity of said lipoxygenase is about 1,400 S.U./liter of substrate.

8.   A method according to claim 4, wherein the concentration of said substrate is between about 1.0×10^{-4} mol/liter and 4×10^{-4} mol/liter.

9.   A method according to claim 3, wherein step c) comprises measuring spectrophotometrically the amount of acid hydroperoxide produced in a fixed incubation period.

10.   A method according to claim 9, wherein said fixed incubation period is effected by stopping the lipolytic reaction in step b) by adding an acid/alcohol mixture.

11.   A method according to claim 9 or 10, wherein said fixed incubation period is from about 5 to about 15 minutes.

12.   A method according to claim 9, wherein the spectrophotometric measuring comprises colorimetric measuring.

13.   A method according to claim 12, wherein the colorimetric measuring employs the capability of the acid hydroperoxide to oxidize ferrous ion to ferric ion.

14.   A method according to claim 9, wherein the spectrophotometric measuring comprises UV spectrophotometric measuring.

15.   A method according to claim 3, wherein said measuring in step c) comprises kinetically measuring the rate of oxygen consumption in step b).
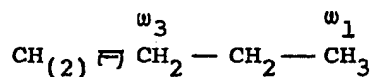
16.   A method according to claim 3, wherein said substrate is based on naturally occurring fatty acids and η is 1, 3 or 5.

17.   A method according to claim 3, wherein said substrate is based on a synthetic fatty acid and η is 1, 2, 3, 4 or 5.

18.   An analytical procedure for the determination of lipase activity comprising adding an unknown sample fluid to a substrate reagent comprising an emulsion of trilinolein, adding lipoxygenase, in the presence of oxygen, said lipoxygenase being added in an amount sufficient to insure that lipolysis is the rate limiting reaction and thereafter calculating lipase activity on the basis of production of acid hydroperoxide.

19.   A method according to claim 18, carried out at a pH of about 8.8 and at a temperature of about 30°C.

20.   A method according to claim 19, wherein the activity of said lipoxygenase is at least about 1,000 S.U./liter of substrate.

21.   A method according to claim 18, wherein said trilinolein is present in a concentration of between about 1.0×10^{-4} mol/liter and about 4×10^{-4} mol/liter.

22.   A method according to claim 18, wherein the production of acid hydroperoxide is determined by spectrophotometry.

23.   A method according to claim 18, wherein the production of acid hydroperoxide is measured kinetically by monitoring the accompanying decrease in oxygen tension.

24.   In a test for lipase activity wherein the triglyceride substrate in emulsified form is contacted with a test sample

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and lipase activity is measured as a function of the product

formed by lipolysis, the improvement comprising:

employing a substrate having the general formula:

$$
\begin{array}{c}
\text{O} \\
\parallel \\
H_2^{\alpha}C - O - C - R_1 \\
(1)
\end{array}
$$

$$
R_2 - \overset{\text{O}}{\overset{\parallel}{C}} - O - \overset{\beta}{CH} \\
(2)
$$

$$
H_2^{\alpha}C - O - C - R_1 \\
(3) \quad \parallel \\
\text{O}
$$

wherein R_1 is $(CH_2)_\eta - (CH_2 \rightleftharpoons \overset{\omega 12}{\underset{\omega 15}{CH}}_{(2)} - CH_2)_2 - (CH \overset{\omega 6}{\underset{\omega 9}{=}} CH - CH_2)_2 -$

$$
CH_{(2)} \rightleftharpoons \overset{\omega 3}{CH_2} - CH_2 \overset{\omega 1}{-} CH_3
$$

η is 1, (2), 3, (4), 5, and

R_2 is a member selected from the group consisting of R_1 and

saturated or (poly)unsaturated fatty acid alkyl chains; and

further employing a sufficient amount of lipoxygenase, in

the presence of oxygen, to ensure that the conversion of sub-

strate to fatty acid by lipolysis is the rate limiting

reaction, and thereafter determining lipase activity as a

function of the acid hydroperoxide produced.

25.   A test according to claim 24, wherein said substrate

is trilinolein.

26.   A test according to claim 24, wherein said test

sample, substrate and lipoxygenase are allowed to react

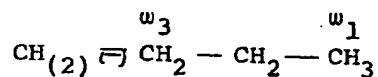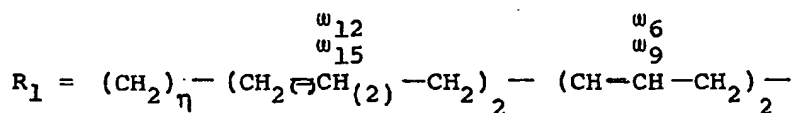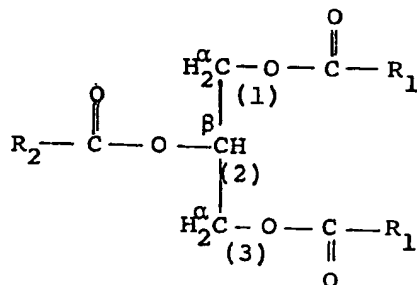at about 30°C. and at a pH of about 8.8.

27.   A test according to claim 24, wherein the activity of said lipoxygenase is at least about 1,000 S.U./liter.

28.   A reagent for determining the lipase activity of a biological fluid comprising

an emulsified substrate effective in the presence of lipase to produce fatty acid by lipolysis, and lipoxygenase in an amount effective, in the presence of oxygen, to convert fatty acid produced by lipolysis to its acid hydroperoxide at a rate sufficient to ensure that lipolysis is the limiting reaction.

29.   A reagent according to claim 28, wherein said substrate is trilinolein.

30.   A reagent according to claim 29, wherein said lipoxygenase has an activity of about 16,800 I.U./ml at 25°C.

31.   A test system for determining the lipase activity of a biological fluid comprising

an emulsified substrate effective in the presence of lipase to produce fatty acid by lipolysis; lipoxygenase in an amount effective, in the presence of oxygen, to convert the acid produced by lipolysis to its acid hydroperoxide at a rate sufficient to ensure that lipolysis is the limiting reaction; and means for measuring the production of acid hydroperoxide as a measure of the lipase activity of the biological fluid.

32.   A test system according to claim 31, wherein said substrate is trilinolein.

33.   A test system according to claim 31 or 32, wherein said means comprises spectrophotometric measuring means.

34.   A test system according to claim 31 or 32, wherein said means comprises kinetic assay means for kinetically measuring the rate of oxygen consumption.

35.   A method for the specific determination of lipase activity in a biological fluid constituting a hetero-geneous system comprising:

(a)   adding a sample of a biological fluid sample to an emulsified substrate containing at least 1.0×10^{-4} mole per liter of a water-insoluble triglyceride having the structural formula:

$$H_2^{\alpha}C-O-\overset{\overset{\displaystyle O}{\|}}{C}-R_1 \quad (1)$$

$$R_2-\overset{\overset{\displaystyle O}{\|}}{C}-O-\overset{\beta}{C}H \quad (2)$$

$$H_2^{\alpha}C-O-\overset{}{\underset{\underset{\displaystyle O}{\|}}{C}}-R_1 \quad (3)$$

$$R_1 = (CH_2)_{\eta}-(CH_2 \overset{\omega 12}{\underset{\omega 15}{-}}CH_{(2)}-CH_2)_2 - (CH\overset{\omega 6}{\underset{\omega 9}{-}}CH-CH_2)_2 -$$

$$CH_{(2)} \overset{\omega 3}{-} CH_2 - CH_2 - \overset{\omega 1}{C}H_3$$

η is 1, (2), 3, (4), 5, and

R_2 = a member selected from the group consisting of R_1 and

saturated or (poly)unsaturated fatty acid alkyl chains;

and wherein said substrate also contains a bile salt;

(b) simultaneously, or immediately thereafter, adding an amount of lipoxygenase high enough to convert the acid produced by lipolysis, in the presence of oxygen, to its acid hydroperoxide at a rate sufficient to ensure that lipolysis is the limiting reaction but low enough to ensure that the rate of reaction with said substrate itself remains low; and

(c) measuring the production of said acid hydroperoxide.

36.   A method according to claim 1,2 or 3, wherein said fatty acid produced by lipolysis comprises linoleic acid.

37.   A method according to claim 18, 19 or 20, wherein said acid hydroperoxide comprises linoleic acid hydroperoxide.

38.   A test according to claim 24, 25 or 26, wherein said fatty acid comprises linoleic acid.

39.   A reagent according to claim 28, 29 or 30, wherein said substrate is effective in the presence of lipase to produce linoleic acid by lipolysis.

40.   A test system according to claim 31 or 32, wherein said substrate is effective in the presence of lipase to produce linoleic acid by lipolysis.